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Streptomyces hygroscopicus Has Two Glutamine Synthetase Genes

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Streptomyces hygroscopicus, which produces the glutamine synthetase inhibitor phosphinothricin, possesses at least two genes (*glnA* and *glnB*) encoding distinct glutamine synthetase isoforms (GSI and GSII). The *glnB* gene was cloned from *S. hygroscopicus* DNA by complementation in an *Escherichia coli* glutamine auxotrophic mutant (*glnA*). *glnB* was subcloned in *Streptomyces* plasmids by insertion into pIJ486 (pMSG3) and pIJ702 (pMSG5). Both constructions conferred resistance to the tripeptide form of phosphinothricin (bialaphos) and were able to complement a glutamine auxotrophic marker in *S. coelicolor*. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of *S. lividans*(pMSG5) revealed a highly overexpressed 40-kilodalton protein. When GS was purified from this strain, it was indistinguishable in apparent molecular mass from the 40-kilodalton protein. The nucleic acid sequence of the cloned region contained an open reading frame which encoded a protein whose size, amino acid composition, and N-terminal sequence corresponded to those of the purified GS. *glnB* had a high G+C content and codon usage typical of streptomycete genes. A comparison of its predicted amino acid sequence with the protein data bases revealed that it encoded a GSII-type enzyme which had previously been found only in various eucaryotes (47 to 50% identity) and nodulating bacteria such as *Bradyrhizobium* spp. (42% identity). *glnB* had only 13 to 18% identity with eubacterial GSI enzymes. Southern blot hybridization experiments showed that sequences similar to *glnB* were present in all of the five other *Streptomyces* species tested, as well as *Frankia* species. These results do not support the previous suggestion that GSII-type enzymes found in members of the family *Rhizobiaceae* represent a unique example of interkingdom gene transfer associated with symbiosis in the nodule. Instead they imply that the presence of more than one gene encoding GS may be more common among soil microorganisms than previously appreciated.

Glutamine synthetase (GS), a pivotal enzyme for nitrogen metabolism, is found in at least three distinct forms. In studies reported to date, one of these forms, GSI, has been primarily associated with procaryotes and another, GSII, has been associated with eucaryotes. A third type of GS has been recently found in the anaerobe *Bacteriodes fragilis* (22). These three types of enzymes are distinct in their primary as well as tertiary structures. GSI is composed of 12 subunits (443 to 474 amino acids each); GSII has 8 subunits (332 to 378 amino acids each) (see Table 2 for references); and the *Bacteriodes fragilis* enzyme has 6 subunits (729 amino acids) (22). Sequence alignments show that the GSI and GSII families are only 15% identical (40) and that GSI has an extended C terminus (see Fig. 6 for references), which includes an adenylation site important for the post-translational control of activity (43). Thermolability of GSII has been widely used to differentiate it from GSI (13, 16, 17).

The strict association of one of these two enzyme families with procaryotes and the other with eucaryotes was first called into question by studies of nitrogen metabolism in nodulating bacteria such as *Rhizobium* (13, 18, 33), *Agrobacterium* (17), *Bradyrhizobium* (7), and *Frankia* (16) species. Biochemical studies demonstrated two isoforms of GS which were independently regulated with respect to the

availability of carbon (12), nitrogen (8, 17, 33), or oxygen (12, 33, 39). Paradoxically, mutations in either one of these genes did not result in glutamine auxotrophy or avirulence (8, 41). Nucleotide sequence information indicated that in *Bradyrhizobium japonicum*, GSI was similar to other procaryotic *glnA*-encoded enzymes (7), while the second enzyme belonged to the family of GSII enzymes (6). Carlson and Chelm interpreted this to mean that the gene encoding GSII had been transferred from plants to bacteria in the nodule (6).

We have been studying nitrogen metabolism in the actinomycete *Streptomyces hygroscopicus*, a soil microorganism which synthesizes phosphinothricin (PPT), an inhibitor of both GSI and GSII (2, 28). PPT is incorporated into a tripeptide (bialaphos) which contains two alanine residues (2, 28). Activation of GS-inhibitory activity in plants or bacteria depends on the activity of peptidases which release PPT. Both PPT and its demethylated analog (DMPT), an intermediate in the biosynthetic pathway (24), are inhibitors of GS activity in *S. hygroscopicus* (S. Imai, personal communication). Since inhibition of GS could be self-limiting for antibiotic biosynthesis, the GS gene was cloned in the expectation that its gene product could be overexpressed to allow PPT or DMPT tolerance and increased production of bialaphos.

Purification and N-terminal sequence analysis of the only GS activity detected in *S. hygroscopicus* grown in rich medium indicated that it belonged to the GSI family (29a). An experiment designed to clone the corresponding gene by

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complementation of an *E. coli glnA*-defective mutant resulted in the isolation of a gene which encoded a GSII-type enzyme.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. coelicolor* A3(2), *S. fradiae* ATCC 10745, *S. glaucescens* ETH22794, and *S. lividans* TK24 (derived from *S. lividans* 66 [32]), as well as plasmids pIJ702 (27) and pIJ486 (50), were obtained from the John Innes Culture Collection. *S. hygroscopicus* SF1293 (ATCC 21705) was obtained from the Meiji Seika Culture Collection. *S. viridochromogenes* JCM 4977, obtained from the Japanese Collection of Microorganisms, is the same as Tü494 (2). *Frankia* sp. strain ArI3 (HFP013003) genomic DNA was kindly provided by P. Simonet. Glutamine-auxotrophic mutants of *S. coelicolor* (strain FS10) and *E. coli* (strain YMC11 [1]), as well as a plasmid containing the *S. coelicolor glnA* gene (pLEW3), were obtained from S. Fisher.

Media. Most of the media and culture conditions for *E. coli* (34) and *Streptomyces* species (23) have been previously described. *E. coli* was grown in solid or liquid medium based on LB broth (34) (containing 50 µg of ampicillin per ml) or W-salts (supplemented as required with 50 µg of glutamine per ml). The minimal medium used for *S. coelicolor* was MM (23) supplemented with histidine (2 µg/ml), uracil (2 µg/ml), and, as required, glutamine (50 µg/ml). Minimal medium for *S. lividans* and *S. hygroscopicus* contained the following (per liter): 7.5 g of soluble starch, 0.75 g of KH_2PO_4 , 25 mg of MgSO_4 , 1.3 g of $\text{NaNO}_3 \cdot 10\text{H}_2\text{O}$, and 10 ml of trace elements solution [containing (per liter) 40 mg of ZnCl_2 , 200 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 10 mg of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 10 mg of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, and 10 mg of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 3\text{H}_2\text{O}$]. YEME (23) or S1 (38) medium supplemented with 10 µg of thioestrepton per ml (kindly supplied by S. J. Lucania, E. R. Squibb & Sons) was used for streptomycete liquid cultures.

DNA manipulations. (i) **DNA isolation and cloning.** DNAs were prepared as previously described for *E. coli* (34) or streptomycetes (23, 37). Restriction enzymes (Takara Shuzo, Ltd.), T4 DNA ligase (Takara Shuzo, Ltd.), and calf intestinal alkaline phosphatase (Toyobo K. K.) were used as recommended by the suppliers.

(ii) **DNA sequencing.** DNA was cloned in M13mp18 or M13mp19 (52), random endpoint deletions were made with exonuclease III (21) (Takara), and sequencing was done by using dideoxy-chain termination reactions (42) (United States Biochemical Corp.). The reactions were analyzed by using the Fuji Gensor Gel System (Fuji Film K. K.), and the DNA sequence was completed on both strands.

(iii) **Southern blot hybridizations.** Genomic DNA (1 µg) was digested with *Bam*HI, separated by agarose gel electrophoresis, and transferred to nitrocellulose filters (34). Filters were washed for 1 h (this and all subsequent steps were carried out at 70°C) in prehybridization solution containing 3× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 4× Denhardt solution (34), and 10 µg of salmon sperm DNA per ml. Probe DNA labeled by nick translation with [^{32}P]CTP (Amersham Corp.) and a kit from Pharmacia was hybridized to the blot overnight. The filters were washed twice for 30 min in 2× SSC–0.1% sodium dodecyl sulfate (SDS). Hybridizing bands were visualized by autoradiography.

Purification of GS. GS (assayed by the method of Bender et al. [3]) was purified from *S. lividans*(pMSG5). A seed

culture was grown for 48 h at 30°C in S1 medium containing thioestrepton, and 1 ml of this culture was used to inoculate each of three 500-ml flasks containing 80 ml of YEME. After growth at 30°C for 72 h, mycelia were collected by centrifugation, washed in 50 mM Tris hydrochloride (pH 7.5), suspended in buffer I (20 mM imidazole, 1 mM MnCl_2), and disrupted by sonication. The sonic extract was clarified by centrifugation ($17,000 \times g$ for 15 min at 4°C), dialyzed against the same buffer, and then applied to a MonoQ (Pharmacia) anion-exchange column. After elution in buffer I containing a gradient of 0 to 1 M NaCl, active fractions were pooled, dialyzed against buffer I containing 0.2 M NaCl, and then applied to a Superose (Pharmacia) molecular size exclusion column equilibrated in the same buffer. Active fractions were pooled and analyzed for purity by SDS-polyacrylamide gel electrophoresis (PAGE).

Protein analysis. (i) **SDS-PAGE.** SDS-PAGE was carried out as described by Laemmli (30). Mycelia were suspended in 1% SDS–10% mercaptoethanol and boiled for 5 min. Protein molecular weight markers were supplied by Bio-Rad Laboratories (SDS-PAGE low-molecular-weight protein standards).

(ii) **N-terminal amino acid analysis.** N-terminal amino acid sequence analysis was performed by Edman degradation, using a gas-phase sequenator (model 470kA; Applied Biosystems, Inc.).

(iii) **Amino acid composition.** Acid hydrolysis of lyophilized protein was carried out in three stages by treating the material first (at 120°C for 40 h) with 6N HCl and then twice (at 150°C for 1 h followed by 150°C for 3 h) with concentrated HCl-fluoroacetic acid (2:1).

RESULTS

Cloning a GS gene from *S. hygroscopicus*. A GS gene (*glnB*) was isolated from *S. hygroscopicus* (Fig. 1). Genomic DNA was inserted into pUC18 and used to transform *E. coli* YMC11 (1). Transformants were plated on LB medium supplemented with glutamine, thiamine, and ampicillin. Colonies were replicated to M9 medium supplemented with thiamine and ampicillin but lacking glutamine. In a population of about 50,000 transformants, 10 glutamine-prototrophic transformants were isolated. Plasmids in each of these isolates contained an insert of 3.7 kilobases. One of these, pMSG1, was further characterized. The region which complemented the *glnA* mutation, presumably a gene encoding GS, was further localized to a 1.6-kilobase fragment by deletion of one end of the insert to generate pMSG2. The observation that the same fragment cloned in the other orientation with respect to the *lac* promoter in pUC19 did not allow growth in the absence of glutamine suggested that the gene was not expressed from its own promoter in *E. coli*.

Expression of *glnB* in *Streptomyces* species. We subcloned the *glnB* gene into the vector pIJ486 to study its expression in streptomycetes. A 1.6-kilobase fragment was first subcloned from pMSG2 into pIJ486 to generate pMSG3 by using the prototrophic strain *S. lividans* 66. The entire insert of pMSG3 was then subcloned into pIJ702 to generate pMSG5.

When pMSG3 and pMSG5 were used to transform a glutamine auxotroph, *S. coelicolor* FS10, both plasmids allowed growth on minimal media in the absence of glutamine. Also, either plasmid allowed *S. lividans* to grow on high concentrations of bialaphos. *S. lividans* is normally sensitive to 1 µg of bialaphos per ml on solid minimal medium; either plasmid allowed growth on 1,000 µg of bialaphos per ml. These observations show that pMSG3 and

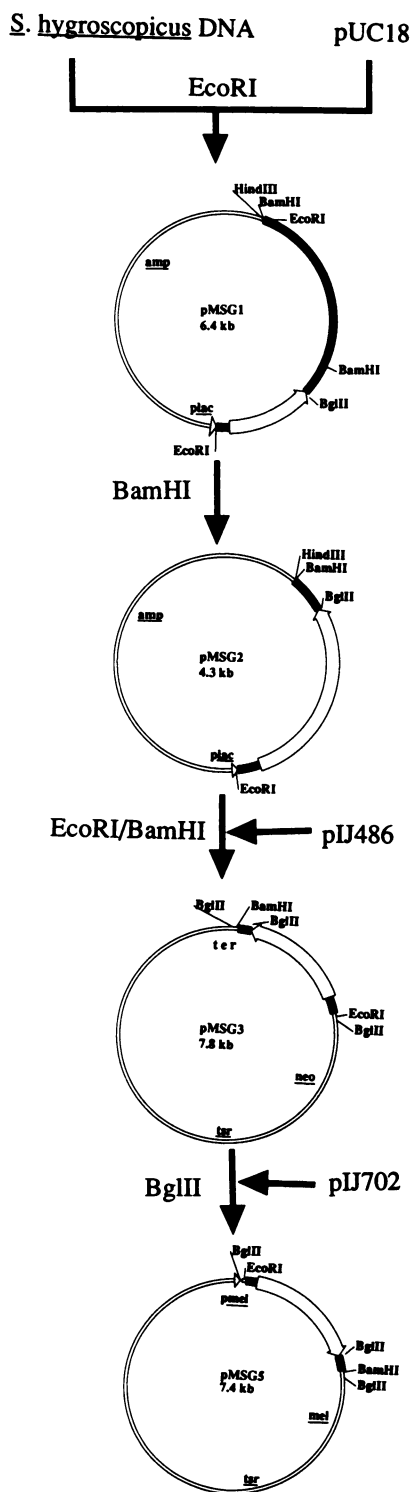


FIG. 1. Construction of plasmids used to isolate and express the *glnB* gene. Enzymes and plasmids used in each cloning step are indicated. The black region indicates *S. hygroscopicus* DNA. The arrows indicate the position of *glnB* which is potentially transcribed from the lactose promoter (*lac*) or melanin (*mel*) promoter (*pmel*). The thiostrepton resistance (*tsr*) or ampicillin resistance (*amp*) genes allowed selection of transformants. The promoter probe vector pIJ486 contained a neomycin resistance gene (*neo*) and fd terminator (*ter*).

pMSG5 encode a GS activity. The bialaphos resistance phenotype was due to either the high-level expression of the GS gene, which titrated out the inhibitor, or the higher resistance of the *glnB* gene product to PPT.

Characterization of the *glnB* gene product. To detect the protein encoded by the cloned gene, we analyzed mycelia by SDS-PAGE. Cultures of *S. lividans*(pMSG5) were grown to late logarithmic phase in YEME, a nitrogen-rich complex liquid medium. Crude extracts were prepared and analyzed by SDS-PAGE (Fig. 2), revealing the presence of a dramatically overproduced insert-specific protein of ca. 40 kilodaltons (kDa).

When GS was purified from this strain (to greater than 95% homogeneity; [Fig. 2; Materials and Methods]), it was identified as the 40-kDa protein. Other bacterial GS proteins, including the major activity present in *S. hygroscopicus* (29a), are much larger (ca. 59 kDa). Furthermore, although the N-terminal sequences of *S. coelicolor* GSI and *S. hygroscopicus* GSI (subsequently designated GSI-Sh) proteins are very similar and resemble other bacterial GS enzymes (GSI), the N-terminal sequence of the 40-kDa protein (subsequently designated GSII-Sh) indicated that it was different (Table 1).

GSI-Sh and GSII-Sh were also differentiated by their relative thermostabilities. Purified GSI-Sh and GSII-Sh were assayed for activity after a 1-h incubation at various temperatures (Table 2). Under these conditions, both enzymes were stable at 30 and 40°C. GSI-Sh was more thermostable than GSII-Sh at 50 and 60°C.

Nucleotide sequence analysis. To characterize the gene encoding GSII-Sh, we sequenced a portion of the 1.6-kilobase fragment by the dideoxy technique of Sanger et al. (42). The sequence (Fig. 3) had a very high G+C content (71%). A potential coding sequence was found by using a program developed by Staden and McLachlan (45), employing a library of codons used in streptomycete structural genes (C. J. Thompson, unpublished data). This open reading frame encoded a gene product of 36 kDa.

Several analyses of the purified GS confirmed that it was encoded by this open reading frame. The total amino acid composition of the purified protein agreed with that predicted by the nucleotide sequence (Fig. 4). The N-terminal

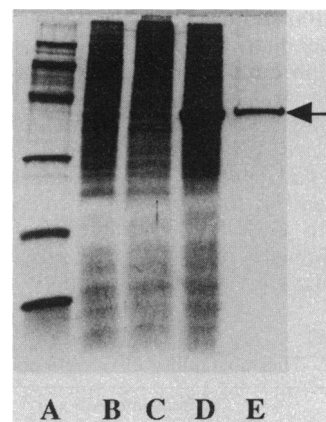


FIG. 2. SDS-PAGE analysis of GSII-Sh expressed in *S. lividans*. Crude cell extracts were prepared as described in Materials and Methods. Lanes: A, molecular mass standards (phosphorylase *b*, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21 kDa; lysozyme, 14 kDa); B, *S. lividans*; C, *S. lividans*(pIJ702); D, *S. lividans*(pMSG5); E, GS purified from *S. lividans*(pMSG5) (arrow).

TABLE 1. Comparison of the N-terminal amino acid sequences of *S. hygroscopicus* GSII, *S. hygroscopicus* GSI, and *S. coelicolor* GSI

Enzyme	Sequence ^a	Reference
<i>S. hygroscopicus</i> GSII	S I K A E Y I W I D G T Q P T A K L R S	
<i>S. hygroscopicus</i> GSI	M W Q N A (D E) A K ? W I A (D E)	29a
<i>S. coelicolor</i> GSI	M W Q N A D D V K K Q I A D E	51

^a Parentheses indicate ambiguous order.

sequence began with the second amino acid predicted by the nucleotide sequence of the structural gene which initiated translation at a GTG and was preceded by a G+A-rich sequence characteristic of ribosome-binding sites. The sequences following the open reading frame featured repeated sequences (Fig. 3) which may be involved in transcriptional termination.

Similarity of *glnB* to sequences in other actinomycetes. Southern blot hybridizations were done to determine whether *glnB* or related sequences could be detected in other actinomycetes including *Frankia* and *Streptomyces* species (Fig. 5). *S. hygroscopicus* and the only other reported bialaphos producer, *S. viridochromogenes*, as well as the nonproducers *S. lividans*, *S. coelicolor*, *S. fradiae*, and *S. glaucusens*, were screened by using a *glnB* probe. All *Streptomyces* species contained a single band which hybridized strongly to the probe. A very weak band was detected in the *Frankia* species. When these strains were screened with a *glnA* probe, each *Streptomyces* species contained a single band which was not the same size as that detected with the *glnB* probe; a hybridizing band was not detected in the *Frankia* species.

Similarity of the GSII-Sh to eucaryotic GSs. The amino acid sequence of GSII-Sh was used to screen the PSEQIP protein data base. The best matches were found to a variety of eucaryotic GSs including those of alfalfa (15), pea (48), kidney bean (two root isoforms [19]), and hamster (20). The next most significant match was to *B. japonicum* GSII, the only other known member of the eucaryotic GS family. Each alignment was assessed for its statistical significance by comparing its alignment score with a distribution of scores obtained with randomized sequences having the same amino acid composition (31). This analysis showed that the alignment score of all eucaryotic GS enzymes listed above with GSII-Sh was >100 standard deviation units separated from the mean of the distribution of randomized scores (>10 standard deviation units is statistically significant). In contrast, using the same alignment parameters, procaryotic GS enzymes including those of *Bacillus subtilis*, *Anabaena*, *Thiobacillus ferrooxidans*, *Rhizobium leguminosarum* (GSI), *Salmonella typhimurium*, *Clostridium acetobutylicum*, *Azospirillum brasilense*, and *E. coli* were only 13 to 18% identical to GSII-Sh. In Table 3 we compare the GSII-Sh enzyme with representative procaryotic and eucaryotic-type enzymes. GSII-Sh is highly similar to other

enzymes in the five regions which are conserved in both procaryotic and eucaryotic enzymes (Fig. 6).

DISCUSSION

The observation of GSII exclusively in nodulating bacteria has led most people working in the field to believe that it served a specialized function which was not relevant to free-living procaryotes. Since this concept will continue to direct experimental design in the field of nitrogen metabolism, the issue must now be critically reconsidered in light of our observation that *S. hygroscopicus* (and probably most *Streptomyces* species) has two genes encoding GS.

A large body of literature describing nitrogen metabolism in enterobacteria supported the assumption that nitrogen limitation did not derepress alternative GS genes. In addition, GS has been identified in various bacteria after purification of the GS activity expressed under specific nutritional conditions. Therefore, historical prejudices and the methods used could have imposed a bias on the results.

As an alternative to biochemical isolation, the identification of new GS genes by cloning in *E. coli* may reveal determinants which are cryptic in their native hosts under laboratory conditions. Successful use of this approach may depend on supplying appropriate transcriptional and translational signals in the *E. coli* vector. Genes which complement *E. coli glnA* mutations have been isolated from genomic DNA of a limited number of organisms including *Anabaena*, *Klebsiella pneumoniae*, *C. acetobutylicum*, *Bacterioides fragilis*, *R. leguminosarum*, *S. coelicolor*, and *T. ferrooxidans*. With the exception of *R. meliloti*, all of these genes encoded GSI-type enzymes. These studies revealed

TABLE 3. Comparison of the amino acid sequences of the *S. hygroscopicus* GSII and other GS enzymes

Origin	Amino acids		Reference
	Total no.	% Identical	

GSI type			
<i>Bacillus subtilis</i>	445	14	46
<i>Anabaena</i> sp. strain 7210	474	16	49
<i>Rhizobium leguminosarum</i>	469	16	10
<i>Clostridium acetobutylicum</i>	444	16	26
<i>Thiobacillus ferrooxidans</i>	468	17	40
<i>Salmonella typhimurium</i>	469	18	25
<i>Streptomyces coelicolor</i>	469	18	51
<i>Azospirillum brasilense</i>	468	17	5
<i>Escherichia coli</i>	472	18	9
GSII type			
<i>Bradyrhizobium japonicum</i> GSII	329	42	6
Chinese hamster	373	47	20
Kidney bean	356	47	19
Pea	357	49	48
Alfalfa	356	50	15
<i>Streptomyces hygroscopicus</i> GSII	337	100	

TABLE 2. Thermostability of GSI-Sh and GSII-Sh

Enzyme	% Remaining GS activity at ^a :				
	4°C	30°C	40°C	50°C	60°C
GSI-Sh	100	95	100	110	40
GSII-Sh	100	105	90	10	5

^a Purified GS enzymes (1 µg) were incubated in buffer I at the temperature indicated for 1 h and then assayed. Activities are expressed as a percentage of the control sample, which was incubated at 4°C.

EcoRI
 1 GAATTCCTTGAAACGGCTGGATGTCGGCTCGGTAACCTGTGGTTACAAACGGGCAACGGACGAGAAATGGCGCGTT

80 GCGAGAGTGCAGGAGTACGCCCGCACCGTACCCGCACCGACCGTGAAGAGGACCCCGTG AGC ATC AAG GCC GAG
 (met) ser ile lys ala glu

154 TAC ATC TGG ATC GAC GGC ACG CAG CCG ACC GCC AAG CTC CGC TCC AAG ACC AAG ATC CTG
tyr ile trp ile asp gly thr gln pro thr ala lys leu arg ser lys thr lys ile leu

214 TCC GAC GGC AGC CGG CTT CCG CGG TGG GGC TTC GAC GGT TCC AGC ACC AAC CAG GCC GAA
 ser asp gly ser arg leu pro arg trp gly phe asp gly ser ser thr asn gln ala glu

274 GGC CAC GCC TCG GAC CTC GTA CTG GAG CCG GTG TTC AGC TGC CCG GAC CCG ATC CGC GGC
 gly his ala ser asp leu val leu glu pro val phe ser cys pro asp pro ile arg gly

334 GGC GAC CAC CTG CTG GTG CTG TGC GAG GTG CTG CAC ACC GAC CTC ACC CCG CAC CCC TCC
 gly asp his leu leu val leu cys glu val leu his thr asp leu thr pro his pro ser

394 AAC ACC CGG GCG CTG CTG CGC CCG GTC GCG GAG CGG TTC GCC GGC CAG GAG CCG ATC TTC
 asn thr arg ala leu leu arg pro val ala glu arg phe ala gly gln glu pro ile phe

454 GGC ATC GAG CAG GAG TAC ACC TTC CTC AAG GGC GAC CGC CCG CTC GGC TTC CCC GAG GGC
 gly ile glu gln glu tyr thr phe leu lys gly asp arg pro leu gly phe pro glu gly

514 GGC GGC TAC CCG GCC CCG CAG GCC GAC TAC TAC TGC GGC GTG GGC GCC GAC GCG ATC TTC
 gly gly tyr pro ala pro gln ala asp tyr tyr cys gly val gly ala asp ala ile phe

574 GGC CGG GAG ATC GTC GAG AAG CAC CTC GAC CTG TGC CTG GCG GCC GGT CTG GGC CTG TCC
 gly arg glu ile val glu lys his leu asp leu cys leu ala ala gly leu gly leu ser

634 GGC ATC AAC GCC GAG GTC ATG CCC GGC CAG TGG GAG TTC CAG GTC GGC GCG CTG CCG CCG
 gly ile asn ala glu val met pro gly gln trp glu phe gln val gly ala leu pro pro

694 CTG GAG GTC TCG GAC CAC ATG TGG GTG GCG CGC TGG CTG CTG CAC CGG GTG GCG GAG GAG
 leu glu val ser asp his met trp val ala arg trp leu leu his arg val ala glu glu

754 TTC GGC GTC ACC GCG TCG CTG GAC GCC AAG CCG GCC AAG GGC GAC TGG AAC GGC GCG GGC
 phe gly val thr ala ser leu asp ala lys pro ala lys gly asp trp asn gly ala gly

814 GCG CAC ACC AAC TTC TCC ACC CGC GCG ATG CGC GAG GGC TAC GAC CCG ATC ATC ACC GCC
 ala his thr asn phe ser thr arg ala met arg glu gly tyr asp pro ile ile thr ala

874 TGC GAG GCG CTG GGC CAG GAC GAC AAG CCG CTG GAG CAC GTC CGC CAG TAC GGC ACC GGC
 cys glu ala leu gly gln asp asp lys pro leu glu his val arg gln tyr gly thr gly

934 ATC GAG GAC CGG CTG ACC GGC GCG CAC GAG ACC GCC CCC TGG GAC GCG TAC TCC TAC GGC
 ile glu asp arg leu thr gly ala his glu thr ala pro trp asp ala tyr ser tyr gly

994 GCC TCC GAC CGC GGC GCC TCG GTG CGC ATC CCC TGG CAG GTC GAG GTC GAG AAG AAG GGC
 ala ser asp arg gly ala ser val arg ile pro trp gln val glu val glu lys lys gly

1054 TAC ATC GAG GAC CGG CGC CCG AAC GCC AAC GTC GAC CCG TAC GTG GTC ACC CGG CTG ATG
 tyr ile glu asp arg arg pro asn ala asn val asp pro tyr val val thr arg leu met

1114 GTG GAC ACC TGC TGC ACG GAG CTG GCG CGG CGC GAG CAG ATC TGACGCCTGCGGTACAGTGGCC
 val asp thr cys cys thr glu leu ala arg arg glu gln ile OPA

1178 GTGCGGTCACGTGACCGCACGGTCGCGCGAG

FIG. 3. Nucleotide sequence of the *glnB* gene. Lines with one arrowhead indicate the positions of imperfect inverted repeats. Lines with two arrowheads indicate imperfect direct repeat sequences. The N-terminal amino acid sequence determined by Edman degradation is underlined. A potential ribosome-binding site is indicated by a dashed line (nucleotides 124 to 131). The sequence has been assigned the GenBank/EMBL accession number M33783.

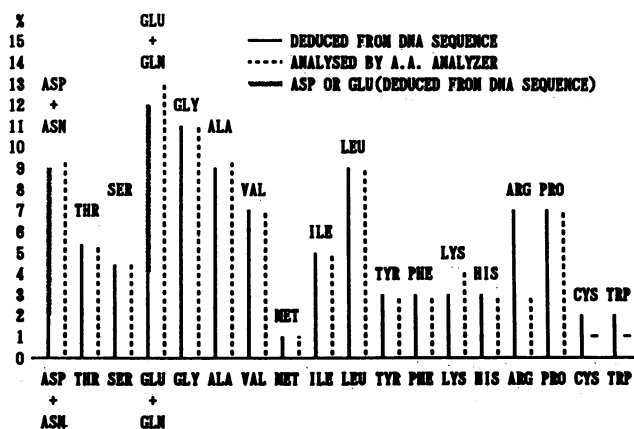


FIG. 4. Amino acid composition of purified GSII-Sh compared with that predicted by the nucleotide sequence. Results are expressed as a percentage of the total composition.

that in addition to GSI and GSII, this strain contained a third GS gene (14). We have used the same approach to identify an *S. hygroscopicus glnB* gene which had not been revealed by biochemical studies (29a). This raised the question of whether the second GS gene was related to the production of PPT or played a more general role in nitrogen metabolism.

Streptomyces species often have target sites which are sensitive to the antibiotics that they produce, and they possess specific mechanisms to avoid the toxicity of their own products (11). This is often achieved by replacement of the target site of the antibiotic with a more resistant analog. Although GSII-Sh is substantially more resistant to PPT in vitro (data not shown) and confers resistance to bialaphos in *S. lividans* and *S. coelicolor*, this is probably not its major function in *S. hygroscopicus* (at least in nitrogen-rich medium). Instead, the gene encoding DMPT/PPT-acetyltransferase (*bar*) seems to be the major resistance determinant; mutagenesis of *bar* results in sensitivity to bialaphos (29). This suggested that *glnB* might not be limited to PPT-producing strains.

To address this question, we carried out Southern hybridizations by using probes for *S. coelicolor glnA* or *S. hygroscopicus glnB*. Each *Streptomyces* species tested, bialaphos producers and nonproducers alike, contained sequences corresponding to *glnA* and to *glnB*. Although hybridization results suggest that both *glnA*- and *glnB*-related genes are present in many *Streptomyces* species, it remains to be demonstrated directly that these are functional genes.

Although GSII could not be detected in *S. hygroscopicus* growing in nitrogen-rich medium during the stationary phase when bialaphos is being produced (29a), the *glnB* structural gene is not defective. The cloned gene complemented a glutamine-auxotrophic marker in *S. coelicolor* and expressed its gene product at strikingly high levels when cloned in *S. lividans*. We have not determined whether the *glnB* structural gene is being expressed from its own or a plasmid-encoded promoter. Future studies of *glnB* regulation will be directed toward defining the physiological con-

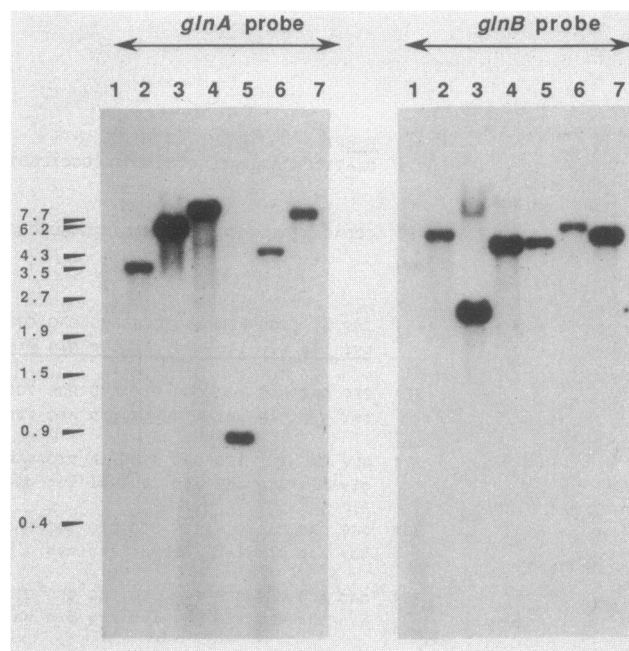


FIG. 5. Detection of *glnA*- and *glnB*-like sequences in actinomycetes. Genomic DNA of *Frankia* species (lane 1), *S. glaucescens* (lane 2), *S. fradiae* (lane 3), *S. coelicolor* A3(2) (lane 4), *S. lividans* 66 (lane 5), *S. viridochromogenes* (lane 6), and *S. hygroscopicus* (lane 7) digested with *Bam*HI were screened with a *glnA* probe (a 453-base-pair *Bam*HI-*Bgl*II fragment isolated from pLEW3) or a *glnB* probe (a 661-base-pair *Pvu*II-*Mlu*I fragment isolated from pMSG2). Both sequences were entirely internal to coding regions. The positions and sizes (in kilobases) of molecular size markers (bacteriophage λ cleaved with *S*ryI) are indicated by arrows.

ditions (presuming that they exist) which activate its expression.

In free-living members of the *Rhizobiaceae*, *glnII* (*glnB*) is transcriptionally activated under nitrogen- or oxygen-limited growth conditions (35, 44). In *Streptomyces* species such conditions may be associated with the initiation of antibiotic production and/or the sporulation process and thus *glnB* could play a role in these processes under some nutritional conditions.

The recent observation that shuttle plasmids are capable of transfer from *E. coli* to *Streptomyces* species (36) suggests that *glnB* may have been conjugally introduced to *Streptomyces* species from members of the *Rhizobiaceae*. It is also possible that the *glnB* gene we isolated from *S. hygroscopicus* was of plant origin and was transferred first to *Frankia* species in the nodule and later to other actinomycetes in the soil. A comparison of GSII sequences from plants, members of the *Rhizobiaceae*, *Frankia* species, and *Streptomyces* species may clarify this possibility. However, the apparent integration of the *glnB* gene into procaryotic regulatory networks and the observation that the *S. hygroscopicus glnB* gene contains a characteristically biased codon composition which reflects the extraordinary high G+C content of the streptomycete genome (4, 47) make it difficult to accept that

FIG. 6. Similarities of representative GS enzymes. The figure shows alignment of amino acid sequences of six GS proteins: *E. coli* (9) (Ec), alfalfa (15) (Af), *B. japonicum* GSI (7) (BjI; only partial sequence available), *B. japonicum* GSII (BjII), *S. coelicolor* (51) (ScI), and *S. hygroscopicus* (ShII). Amino acids identical to *glnB* are shaded. Dotted boxes indicate five regions conserved among both GSI- and GSII-type enzymes. Large black dots indicate unsequenced regions of the *B. japonicum* gene.

No	GS			
1	Ec	MSAEHV	LTMLNEHEVKFVDRFTDTKGKEQHVTPAHQVNAEFFEKGK	FDGSSIGGWKINE
2	Af	M SLLSDLIN	LDLSETTEKIIETINIGSGLD IISARTIPGPVTPSOI IK	NYDGSSTIGAP ED
3	Bj I	MK TAKDV	LKSIKDNVQYDLRFTIPRGKWRHVTFDVSIMIDEIFAEGTM	FDGSSIAGYKAINEN
4	Bj II		MTKYKLYVILQYTPNIGKQKEFASFPTLEHVEL	FDGSSITQQAEC IS
5	Sc	NFQNAADV	KKFIAEDVKFVDRFCILPGVMQHFTLPATAFDPDAE QA	FDGSSIRGFQAIHE
6	Sh II		SIIKAYIWDGTQPAKIRSKELIS DGSRI PR	FDGSSINQAEC HAS

Region I →

No	GS			
1	Ec	IMMMDASTAVIIFFF	ADSTIIRSDI EPGTLQGYDRDRSMK	AEDYLSTGIADTVLFGPPEFFFLFD
2	Af	EVIIYQA IFKIF	RNNIIMDA YTPAG	EITNKHAAAKIFSHPDVVAEVIWYIIGQYTLQK
3	Bj I	IMCIMP????????
4	Bj II	ICLKPVA VFDAAR	TNGVIMC MNPIC	KIPHASNKAT ILD DAGA WFGFQEV EFKY
5	Sc	IMSRDLSTARVIF	RDKTNINFFI HDPITGEQYSRDRNVAKK	AEAYLASTGIADTAFFGPAFYVFD
6	Sh II	ILYIEVF SCPRI	GGDHILUCLY EHTIL	IPHSNTRALLRPVAER FAGQETIGIIGQYTFEKG

← Region I

No	GS			
1	Ec	IFGSSISGSHVAIDIEGAWNSSTQ	YEGGNK	HRPAVKGGFPVPPV
2	Af	INWELWV	IFGCGGPVGGAG	KA FNDIDSYKACLYAGINISIN
3	Bj I
4	Bj II	IGP IIGPT	S	HYPAFGPVTIVFNSVGDVAKIYEENIDICLAAGINHEGIN
5	Sc	SVFATRENSFYHIDSEAGAWNTG	ALEDNR	YKVRKGGFPVPPV
6	Sh II	IA IIGPT	IGGYPAGADYGGG	IFGFEYKHNIDICLAAGLSCIN

No	GS			
1	Ec	HEVATAAG	NEVATRFNTMTKKADEIQYKYVVNNAHREGKA	TFMPIMFED NGSGMCHMSL
2	Af	GVM IIG	NEVATRFPSVGISAGIEIYRYIEITEVAGVL	SFDPKRIED NCAGAHNYS
3	Bj I
4	Bj II	AEVA KIG	NEVATRFKGSKKAADENMRYMLLTCKYID	EFHCXEL GDTDNNGSEMANTS
5	Sc	HEVGTAG	AEVINYKFTLAAADLQFKYIVKNVWKNKT	TFMPIMFED NGSGMIVHQS
6	Sh II	HEV IIG	NEVATRFALPPIEVSDHMYVWILHVAEFGTA	SLAKTAG NCAGAHNYS

Region II

Region III

No	GS				
1	Ec	SKNGVNLFAGDK YAGLSEQALYYIG	GVIKHAKAINAL	ANPTTNSYKRLVPEYEAPVM	LAYS
2	Af	IKSMDG DEVLKIKK	KHKIAA	EGNE	RNTRRTADINTFLWV
3	Bj I
4	Bj II	IEYMTVG KEYFEALMAFD	NMDIAV	GPND	KIKKKTATNKFYGV
5	Sc	WSGGEPLFYDQGYAGLSDTARYYIG	GILKHAPSLLAF	TNPTVNSYHRLVPEAPVN	LVYSQ
6	Sh II	IRAN	EDPITCQDD	PI IVRO	ITIE DLTAAETAPDAYSGA

Region IV

No	GS			
1	Ec	V VSSPKARR	EVFDPAAVLCFA	ALWAGLDGIKN KIHPGEAMDKNLYDLPPEEAKEIPQVAGSLEE
2	Af	R DTKACKVYF	EDRFSNMVYVS	MIADTILWK P*
3	Bj I
4	Bj II	HS FNNGYQL	EDRFSNQDQVIV	RS*
5	Sc	ITGSNPKARR V	FAEDSGNPLAFS	ALWAGLDGIKN KIHPAEPIDKDYELAPEEHANVAQVPTSLGA
6	Sh II	W QVVEKQV	EDRFSNVDPVYV	IVDCTELARRQI*

Region V

No	GS	
1	Ec	ALNELDLREFLKAGGVFTDEAIDAYIALRRE EDDRVRMTPHPVEFELYYSV*
2	Af	
3	Bj I	
4	Bj II	VLDRLADHEFLQGDVFTPDLIETWIDFKRANEIAPLQLRPHPEFEMYFDV*
5	Sc	
6	Sh II	

the presence of a *glnB* gene in this procaryote necessarily indicates recent genetic transfer from a eucaryote.

It is obvious from our results that the simultaneous presence of two different GS genes among soil microorganisms is more prevalent than was previously appreciated; this has several ramifications. First, in biochemical and genetic studies, the knowledge that more than one GS gene may be present could clarify the interpretation of results. Second, it suggests that *glnB* does not represent a plant gene which was transferred and selected in bacteria for a specialized role in nitrogen fixation. Instead, defining the biochemical significance of these GS isoforms should now be considered a more fundamental problem of bacterial physiology.

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